How does *Epichloë festucae* avoid the host defence response?

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Abstract

Epichloë festucae is a filamentous fungus, which forms symbiotic associations with aerial tissues of *Lolium* and *Festuca* grass species. Chitin, a polymer of N-acetyl-D-glucosamine, is an important component of the fungal cell wall and a well-known pathogen associated molecular pattern (PAMP). Chitin promotes pathogen-triggered immunity (PTI) upon hydrolysis with plant chitinases and release of chitin oligomers. Therefore, to establish a stable and successful symbiosis, the endophyte needs to remain 'hidden' from the host immune system or actively suppress it. Confocal laser scanning microscopy (CLSM)-based analysis of leaf tissue infected with the *E. festucae* wild type strain and infiltrated with the chitin-specific molecular probe, WGA-Alexa Fluor-488, showed that only the septa of endophytic hyphae bound this probe while the entire cell wall was labelled in epiphyllous hyphae confirming previous observations that hyphal cell wall chitin is either masked or remodelled in endophytic hyphae. The aims of this project were (i) to test whether *E. festucae* LysM-containing proteins have a role in binding to or sequestering cell wall chitin oligomers and thereby preventing PAMP-triggered immunity and (ii) to analyse the composition of the cell wall of endophytic and epiphytic hyphae. An analysis of the *E. festucae* genome identified seven genes encoding proteins with LysM domains. Expression of two of these genes, *lymA* and *lymB*, increased *in planta* compared to in culture. Interestingly, both are divergently transcribed from chitinase encoding genes (*chiA* and *chiB* respectively), which also have increased expression *in planta*. Single gene deletion mutants of *lymA, lymB, chiA* and *chiB* as well as a double gene deletion Δ*lymA/B* were generated, and their plant interaction phenotype analysed. Plants infected with Δ*lymA*, Δ*lymB* or Δ*chiA* had the same plant-interaction phenotype as wild type whereas Δ*chiB* and Δ*lymA/B* mutants had defects in hyphal growth within the leaves. Analysis of hyphal cell wall structure using Chitin Binding Protein (CBP) and chitosan (CAP (Chitosan Affinity Protein) and OGA-488)-specific eGFP-based biosensors suggest that cell wall chitin is converted to chitosan in endophytic hyphae. This structural change is consistent with a lack of a defence response when *E. festucae* forms a mutualistic symbiotic association with *L. perenne*. Three *E. festucae* chitin deacetylase genes were identified (*cdaA*, *cdaB* and *cdaC*), and gene expression analysis showed *cdaA* expression is significantly increased *in planta* compare to in culture. Functional analysis of *cdaA* revealed that although plants infected with the Δ*cdaA* mutant had a similar whole plant interaction phenotype as wild type, they had an abnormal

cellular phenotype. Patches of chitin were exposed along the endophytic hyphae confirming this mutant was unable to convert chitin to chitosan. However, hyphae in these plants still labelled with the chitosan biosensor OGA-488 demonstrating that despite the deletion of the *cdaA*, the hyphal cell wall of endophytic hyphae still contain chitosan suggesting that another chitin deacetylase, possibly CdaB has a redundant function in *E. festucae*. Collectively these results show that *lymA*, *lymB* and *chiB* are required for establishment of the symbiosis between *E. festucae* and *L. perenne*. In addition, this study shows that chitin is converted to chitosan in the hyphal cell wall of endophytic hyphae during the infection and colonisation of the host. The *E. festucae* chitin deacetylase gene *cdaA* is also essential for proper hyphal growth *in planta* and the symbiotic interaction.

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Abbreviations

aa Amino acid

Amp Ampicillin

AmpR Ampicillin resistant

ap appressorium

Avr Avirulence

BLAST Basic local alignment search tool

BLASTn Nucleotide database search using a nucleotide query

BLASTp Protein database search using a protein query

bp Base pair(s)

cda chitin deacetylase

cDNA Complementary DNA

chi chitinase

cf conidiophore

CFW Calcofluor white

CLSM Confocal Laser Scanning Microscopy

CWI Cell wall integrity

cu cuticle

DIC Differential interference contrast

DIG Digoxigenin

dir direction

DNA Deoxyribonucleic acid

dNTP Deoxynucleotide triphosphate

EDTA Ethylene diamine tetra-acetic acid

eGFP Enhanced green fluorescent protein

en endophytic hyphae

ep epiphyllous hyphae

ETI effector-triggered immunity

ETS effector-triggered susceptibility

g Gram

gDNA Genomic DNA

Gen Geneticin

GenR Geneticin resistant

GH18 glycoside hydrolase family 18

GFP Green fluorescent protein

GPI Glycosylphosphatidylinositol

gt germ tube

h Hour(s)

Hph Hygromycin

HR hypersensitive response

HygR Hygromycin resistant

IDC Impaired development of crippled growth

ih infection hyphae

kb Kilobase(s)

KO Knock-out

L Litre

LB Lysogeny broth

LysM lysin motif

M Molar

MAPK(K/K) Mitogen activated protein kinase (kinase/kinase)

MAMP Microbe associated molecular pattern

Mb millions of base pairs

mg Milligram

µg Microgram

min Minute(s)

µL Microlitre

mL Millilitre

µm Micrometre

µM Micromolar

mm Millimeter

mM Millimolar

Mo *Magnaporthe oryzae*

MOB monopolar spindle-one-binder

mRNA Messenger ribonucleic acid

Nc *Neurospora crassa*

NCBI National Centre for Biotechnology Information

*ntp*II Geneticin

Nox NADPH oxidase

PAMP Pathogen associated molecular pattern

PCR Polymerase chain reaction

PD Potato dextrose

PEG Polyethylene glycol

pro protoperithecia mutant

PTI PAMP-triggered immunity

PRR Pattern recognition receptors

RG Regeneration

RNA Ribonucleic acid

RNase Ribonuclease

RNA-seq Ribonucleic acid sequencing

rpm Revolutions per minute

RT Reverse transcriptase

RT-PCR Reverse transcriptase-polymerase chain reaction

SAK Stress-activated kinase

SAM Shoot apical meristem

SDS Sodium dodecyl sulfate

Sp spore

STRIPAK Striatin interacting phosphatase and kinase

sv substomatal vesicle

sym Symbiosis mutant

TBE Tris-boric acid-EDTA

tBLASTn Translated nucleotide database search using a protein query

T-DNA Transfer-deoxyribonucleic acid

TEM Transmission electron microscopy

TMD Transmembrane domain

Um *Ustilago maydis*

UV Ultraviolet

V Volts

v/v Volume/volume ratio

WT Wild-type

w/v Weight/volume ratio

YE Yeast extract

°C Degrees Celsius